NUCLEIC ACID SEQUENCES THAT CAN BE USED AS PRIMERS AND PROBES IN THE AMPLIFICATION AND DETECTION OF SARS CORONAVIRUS

The present invention is related to nucleic acid sequences that can be used in the field of virus diagnostics, more specifically the diagnosis of infections with a novel human coronavirus causing Severe Acute Respiratory Syndrome (SARS).

An outbreak of atypical pneumonia, referred to as SARS, was first identified in late 2002 in Guangdong Province, China (Marra *et al.* (1) or Rota *et al.* (2)). After similar cases were detected in patients in Hong Kong, Vietnam, and Canada during February and March 2003, the World Health Organization (WHO) issued a global alert for the illness. By May 22nd, 2003, over 8000 SARS cases and 682 SARS-related deaths were reported to WHO from over 30 countries around the world.

Most of these cases were related to exposure to SARS patients in residential or healthcare settings. The incubation period for the disease usually ranges from 2 to 7 days. Infection is usually characterized by fever and flu-like symptoms, which are followed a few days later by a dry, non-productive cough, and shortness of breath. Death from progressive respiratory failure occurs in about 3% to nearly 10% of cases (3-7).

Conventional diagnostic kits could authorize the detection of antibodies produced in response to the SARS coronavirus infection. Different types of antibodies (IgM and IgG) appear and change in level during the course of infection. They can be undetectable at the early stage of infection. Immunoglobulin G usually remains detectable after resolution of the illness. The following test formats are being developed, but are not commercially available yet:

- Enzyme Linked ImmunoSorbant Assay (ELISA): a test detecting a mixture of IgM and IgG antibodies in the serum of SARS patients yields positive results reliably at around day 21 after the

onset of illness, and

- Immunofluorescence Assay (IFA): a test detecting IgM
antibodies in serum of SARS patients yields positive results
5   after about day 10 of illness; this test format is also
used to test for IgG; this is a reliable test requiring the use
of fixed SARS virus infected cell cultures, studied under an
immunofluorescence microscope.

Positive antibody test results indicate a previous
10   infection with SARS-Coronavirus. Seroconversion from negative
to positive or a four-fold rise in antibody titre from acute to
convalescent serum indicates recent infection. With negative
antibody test results, no detection of antibody after 21 days
from onset of illness seems to indicate that no infection with
15   SARS-Coronavirus took place.

Clearly, the current generation of SARS antibody tests,
which use the immunofluorescence and ELISA techniques, have a
sensitivity problem. Immunofluorescence can only detect the
antibodies after a patient has carried the disease for at least
20   10 days, while for ELISA, about 20 days are needed. During that
time, the patient may have spread the virus to other people. In
that context, the tests are mainly useful after
hospitalisation, for confirming whether a patient has SARS.
That is the reason why these tests are not very useful for
25   infectious disease control. Thus the need exists for a bedside
test, a quick on-the-spot test which allows the detection (from
secretions) at an early stage of the disease or the detection
of early antibodies, say from day five (of infection) onwards.

Another conventional detection method is constituted by
30   cell cultures. Virus in specimens (such as respiratory
secretions, blood or stool) from SARS patients can also be
detected by inoculating cell cultures and growing the virus.
Once isolated, the virus must be identified as the SARS virus
with further tests. Cell culture is a very demanding test, but
35   currently (with the exception of animal trials) the only means

to show the existence of a live virus. Positive cell culture results indicate the presence of live SARS-Coronavirus in the sample tested. Negative cell culture results do not exclude

5 SARS. Moreover these two conventional techniques (detection of antibodies and cell culture) require a laboratory and trained staff as well as time to carry out the test. These tests are good and robust in scientific laboratories but they are not rapid enough to have an efficient diagnosis in due time.

10 Whereas conventional virus diagnosis has been based predominantly on the detection of viral antigens or specific antibodies thereto, in recent years attention has shifted towards methods for the direct and rapid detection of the genome of viruses or nucleic acid sequences derived thereof,

15 both RNA and DNA. In this respect, the very short time-to-result is a crucial factor to opt for nucleic acid detection. These methods are usually based on nucleic acid hybridization. Nucleic acid hybridization is based on the ability of two strands of nucleic acid containing complementary sequences to

20 anneal to each other under the appropriate conditions, thus forming a double stranded structure. When the complementary strand is labeled, the label can be detected and is indicative for the presence of the target sequence. Especially in combination with methods for the amplification of nucleic acid

25 sequences these methods have become an important tool in viral diagnosis.

Nucleic acid amplification techniques are especially useful as an additional technique in cases where serological methods give doubtful results or in cases where there may be a

30 considerable time period between infection and the development of antibodies to the virus. With HIV for example, seroconversion usually can occur some 3-6 weeks after exposure to the virus. Thus, whereas no antibodies will be detected with conventional immunoassays, proviral DNA or circulating viral

35 RNA may already be detectable. Also in monitoring antiviral

therapy, methods based on nucleic acid amplification have several advantages over serological methods. Especially quantitative amplification methods provide a powerful tool in

5   assessing the changes in the amount of virus present before and during therapy.

The choice of the oligonucleotides to be used as primers and probes in the amplification and detection of nucleic acid sequences is critical for the sensitivity and specificity of

10   the assay. The sequence to be amplified is usually only present in a sample (for example a blood sample obtained from a patient suspected of having a viral infection) in minute amounts. The primers should be sufficiently complementary to the target sequence to allow efficient amplification of the viral nucleic

15   acid present in the sample. If the primers do not anneal properly (due to mispairing of the bases on the nucleotides in both strands) to the target sequence, amplification is seriously hampered. This will affect the sensitivity of the assay and may result in false negative test results. Due to the

20   heterogeneity of viral genomes false negative test results may be obtained if the primers and probes are capable of recognizing sequences present in only part of the variants of the virus.

Various techniques for amplifying nucleic acid are known

25   in the art. One example of a technique for the amplification of a DNA target segment is the so-called "polymerase chain reaction" (PCR). With the PCR technique the copy number of a particular target segment is increased exponentially with a number of cycles. A pair of primers is used and in each cycle a

30   DNA primer is annealed to the 3' side of each of the two strands of the double stranded DNA-target sequence. The primers are extended with a DNA polymerase in the presence of the various mononucleotides to generate double stranded DNA again. The strands of the double stranded DNA are separated from each

35   other by thermal denaturation and each strand serves as a

template for primer annealing and subsequent elongation in a
following cycle. The PCR method has been described in Saiki et
al., Science 230, 135, 1985 and in patents EP-B-0.200.362 and
5   EP-A-0.201.184.

Another technique for the amplification of nucleic acid is
the so-called transcription based amplification system (TAS).
The TAS method is described in WO-A-88/10315. Transcription
based amplification techniques usually comprise treating target
10   nucleic acid with two oligonucleotides one of which comprises a
promoter sequence, to generate a template including a
functional promoter. Multiple copies of RNA are transcribed
from said template and can serve as a basis for further
amplification.

15   An isothermal continuous transcription based amplification
method is the so-called NASBA process ("NASBA") as described in
EP-B-0.329.822. NASBA includes the use of T7 RNA polymerase to
transcribe multiple copies of RNA from a template including a
T7 promoter. Other transcription based amplification techniques
20   are described in EP-A-0.408.295. EP-A-0.408.295 is primarily
concerned with a two-enzyme transcription based amplification
method. Transcription based amplification methods, such as the
NASBA method as described in EP-A-0.329.822, are usually
employed with a set of oligonucleotides, one of which is
25   provided with a promoter sequence that is recognized by a DNA
dependent RNA polymerase such as, for example, T7 RNA
polymerase. Several modifications of transcription-based
techniques are known in the art. These modifications comprise,
for example, the use of blocked oligonucleotides (that may be
30   provided with a promoter sequence). These oligonucleotides are
blocked so as to inhibit an extension reaction proceeding there
from (US-A-5,554,516). One or more "promoter-primers"
(oligonucleotides provided with a promoter sequence) may be
used in transcription based amplification techniques,

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overlap with the BNI-1 and BNI-2 sequences (Ksiazek *et al.*
(13)).

When primers specifically targeting the BNI-1 fragment and
5 the CDC fragment were used in a long-range PCR protocol, a
region extending from the CDC fragment to the BNI-1 fragment
was amplified. The obtained fragment had the expected length of
3 kb. It was sequenced from both ends, and the sequences were
found to be identical to the CDC and BNI-1 sequences,
10 respectively, demonstrating that the two sequences were derived
from a contiguous RNA molecule and, thus, from the same virus.
Since the same virus was isolated from two independent cases of
SARS, and since there was serologic evidence of an acute
infection with this virus in the underlying patients, it was
15 considered that the coronavirus might have a role in causing
SARS. Specific diagnostic assays based on RT-PCR were therefore
established for the detection of the pathogen.

However, PCR and RT-PCR also have problems. Drosten *et al.*
(10) developed a diagnostic method for SARS that makes use of a
20 nested set of primers designed within the BNI-1 fragment. The
outer set of primers detected the virus in clinical specimens
from the index patient and different patients, who also had
clinical signs of SARS. Additional specimens were positive on
nested RT-PCR. To have a practical and quantitative test, a
25 real-time RT-PCR with a 5'-nuclease probe was established.
After optimization with the use of quantified RNA transcribed
in vitro, the assay reliably detected 10 copies of RNA per
reaction, corresponding to 830 RNA molecules per millilitre of
specimen. The sensitivities of nested and real-time PCR were
30 equivalent.

The specificity of the PCR with outer primers of the
nested PCR, and of the real-time PCR was tested with the use of
RNA purified from cultures of bovine coronavirus, avian
infectious coronavirus, porcine transmissible gastroenteritis
35 coronavirus and human coronaviruses 229E and OC43. None of the

optionally combined with the use of one or more
oligonucleotides that are not provided with a promoter
sequence. For RNA amplification, a transcription based
5   amplification technique, is a preferred technology.

Amplification using PCR can also be based on an RNA
template. The actual PCR needs to be preceded by a reverse
transcription step to copy the RNA into DNA (RT-PCR). However,
if RT-PCR is used for the detection of viral transcripts,
10   differentiation of mRNA- and DNA-derived PCR products is
necessary. DNAse treatment prior to RT-PCR can be employed
(Bitsch et al. (8); Meyer et al. (9)), but sometimes fails to
remove contaminating DNA sufficiently (Bitsch et al. (8)).
That is the solution advocated by Drosten et al. (10) for the
15   detection of the SARS Coronavirus. Thus by performing random
amplification with fifteen different PCRs under low-stringency
conditions and including, an initial reverse-transcription step
to detect RNA viruses, about twenty distinct DNA fragments were
obtained and sequenced. Three of the fragments did not match
20   any nucleotide sequence in the database
(www.ncbi.nlm.nih.gov:80/BLAST). In a translated BLAST search
these fragments showed homology to coronavirus amino acid
sequences. Two of the fragments were 300 nucleotides in length
and identical in sequence, and the third fragment was 90
25   nucleotides in length (sequences BNI-1 and BNI-2, respectively,
as reported on the Web site of the WHO network on March 25).
Detailed sequence analysis revealed that both fragments were
located in the open reading frame 1b of coronaviruses. The
novel coronavirus isolate was termed FFM-ic (for Frankfurt am
30   Main index case).

Simultaneously, a group at the Centers for Disease Control
and Prevention (CDC), Atlanta, USA identified a 400-nucleotide
coronavirus fragment (Urbani isolate, reported on the Web site
of the WHO network on March 24). This sequence did not show an

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PCR assays cross-reacted with these viruses - a finding that is consistent with their considerable genetic differences from the novel coronavirus. Quantification of the viral RNA

5   concentration in clinical specimens from the index patient and Contact 1 by real-time PCR revealed that the highest concentration - as high as 100 million copies per millilitre - was present in sputum. After enrichment of virus by ultra centrifugation, viral RNA was also detected in the serum of the

10 index patient, indicating the presence of low-level viremia during symptomatic disease. Both the index patient and Contact 1 had viral RNA in stool samples obtained during late convalescence, suggesting that virus may be shed in faeces for prolonged periods of time. To investigate whether the novel

15 coronavirus was prevalent in patients in Germany who had gastrointestinal symptoms, a collection of fifty-four stored stool samples was tested with the use of the real-time RT-PCR assay. None of the samples tested positive.

The established PCR assays have also been used to test

20 respiratory samples from German patients with symptoms and a travel history compatible with SARS. So far, sixty-seven samples from fifty-five patients have been tested. One patient fulfilling the WHO criteria for probable SARS was coronavirus-positive on PCR. PCR protocols, as well as positive control

25 material, have been made available to laboratories worldwide. Until standardized reagents for virus and antibody detection are available and methods have been adequately field tested, SARS diagnosis remains based on the clinical and epidemiological findings: acute febrile illness with

30 respiratory symptoms not attributed to another cause and a history of exposure to a suspect or probable case of SARS or their respiratory secretions and other bodily fluids. Those requirements are reflected in the current WHO case definitions for suspect or probable SARS.

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Researchers in several countries are working towards developing fast and accurate laboratory diagnostic tests for the SARS-Coronavirus. Molecular tests based on PCR
5   amplification are one of the faster and more accurate tests that could achieve this goal.

Polymerase chain reaction (PCR) can detect genetic material of the SARS-Coronavirus in various specimens (blood, stool, respiratory secretions or body tissues). Same primers,
10   which are the key pieces for a PCR test, have been made publicly available by WHO network laboratories on the WHO web site. A ready-to-use PCR test kit containing primers and a positive and negative control has been developed. Testing of the kit by network members is expected to quickly yield the
15   data needed to assess the test's performance, in comparison with primers developed by other WHO network laboratories and in correlation with clinical and epidemiological data. Principally, existing PCR tests are very specific but lack sensitivity. This means that negative tests cannot rule out the
20   presence of the SARS virus in patients. Furthermore, contamination of samples in laboratories in the absence of laboratory quality control can lead to false positive results. Positive PCR results, with the necessary quality control procedures in place, are very specific and mean that there is
25   genetic material (RNA) of the SARS-Coronavirus in the sample. This does not mean that there is live virus present, or that it is present in a quantity large enough to infect another person. Negative PCR results do not exclude SARS. SARS-Coronavirus PCR can be negative for the following reasons:
30   - the patient is not infected with the SARS coronavirus; the illness is due to another infectious agent (virus, bacterium, fungus) or a non-infectious cause,
- the test results are incorrect ("false-negative"). Current tests need to be further developed to improve sensitivity,
35   - specimens were not collected at a time when the virus or its

genetic material was present. The virus and its genetic material may be present for a brief period only, depending on the type of specimen tested,·

5 - specimens were not properly handled prior to nucleic acid extraction and nucleic acids have become deteriorated.

It is the purpose of this invention to provide reagents and methods to realise kits for diagnosing SARS disease earlier than former kits. The use of the oligonucleotides according to

10 the invention is not limited to any particular amplification technique or any particular modification thereof, however these oligonucleotides permit amplification of RNA by using a transcription-based amplification technique, preferably the NASBA. Our primers sets are very efficient compared to the RT-

15 PCR primers. In contrast to RT-PCR, NASBA, which is based on RNA transcription by T7 RNA polymerase (Kievits et al. (11); Compton (12)), does not need differentiation between RNA- and DNA-derived amplification products since it uses RNA as its principal target. RT-PCR for SARS have a too low sensitivity,

20 often nested PCR protocol needed to achieve satisfying sensitivity. The nested protocol is then riskful in terms of contamination, i.e. falses positives. NASBA can achieve sensitivity comparable to nested protocol in a "one-step" amplification avoiding risk for contamination.

25 The oligonucleotides of the present invention can likewise be used in quantitative amplification methods. An example if such quantitative method is described in EP-A-0.525.882. The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or

30 ribonucleotides. Such oligonucleotides may be used as primers and probes.

Of course, based on the sequences of the oligonucleotides of the present invention, analogues of oligonucleotides can also be prepared. Such analogues may constitute alternative

35 structures such as "PNA" (molecules with a peptide-like

backbone instead of the phosphate sugar backbone of normal
nucleic acid) or the like. It is evident that these alternative
structures, representing the sequences of the present invention
are likewise part of the present invention.

The term "primer" as used herein refers to an
oligonucleotide either naturally occurring (e.g. as a
restriction fragment) or produced synthetically, which is
capable of acting as a point of initiation of synthesis of a
primer extension product which is complementary to a nucleic
acid strand (template or target sequence) when placed under
suitable conditions (e.g. buffer, salt, temperature and pH) in
the presence of nucleotides and an agent for nucleic acid
polymerization, such as DNA dependent or RNA dependent
polymerase. A primer must be sufficiently long to prime the
synthesis of extension products in the presence of an agent for
polymerization. A typical primer contains at least about 10
nucleotides in length of a sequence substantially complementary
or homologous to the target sequence, but somewhat longer
primers are preferred. Usually primers contain about 15-28
nucleotides, preferably 20-26 nucleotides, but longer primers
may also be employed, especially when the primers contain
additional sequences such as a promoter sequence for a
particular polymerase.

Normally a set of primers will consist of at least two
primers, one "upstream" primer and one "downstream" primer,
which together define the amplicon (the sequence that will be
amplified using said primers).

Primarily for the use in transcription based amplification
techniques, the oligonucleotides according to the invention may
also be linked to a promoter sequence. The term "promoter
sequence" defines a region of a nucleic acid sequence that is
specifically recognized by an RNA polymerase that binds to a
recognized sequence and initiates the process of transcription
by which an RNA transcript is produced. In principle any

promoter sequence may be employed for which there is a known
and available polymerase that is capable of recognizing the
initiation sequence. Known and useful promoters are those that

5   are recognized by certain bacteriophage RNA polymerases such as
bacteriophage T3, T7 or SP6. Oligonucleotides linked to a
promoter sequence are commonly referred to as "promoter
primers". Their function as a primer, e.g. the starting point
for an elongation reaction, however, may be blocked, as already

10 mentioned above, or absent in some embodiments of transcription
based amplification reactions.

It is understood that oligonucleotides consisting of the
sequences of the present invention may contain minor deletions,
additions and/or substitutions of nucleic acid bases, to the

15 extent that such alterations do not negatively affect the yield
or product obtained to a significant degree. Where
oligonucleotides according to the present invention are used as
probes, the alterations should not result in lowering the
hybridization efficiency of the probe. For example, in case of

20 transcription based amplification techniques, wherein one or
more of the primers may be provided with a promoter sequence,
the introduction of a purine-rich (= G or A) hybridizing
sequence, just after the promoter sequence may have positive
effects on the transcription (when there are C's and T's

25 abortive transcription may occur). If no such sequence is
available in the target nucleic acid a purine-rich sequence can
be inserted in the oligonucleotide just following the last
three G residues of the promoter sequence.

The sequences of the present invention are reflected as

30 DNA sequences. The RNA equivalents of these sequences are
likewise part of the present invention.

An aspect of the invention is a pair of oligonucleotides,
for use as a set in the amplification of a target sequence of
the genome of SARS Coronavirus, said pair consisting of:

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a first oligonucleotide being 10-50 nucleotides in length and
comprising at least a fragment of 10 nucleotides of:
SEQ ID 1: TACCTCTCCA GCTAGGATTT TCTACAGGTG TTAACTTAGT
AGCTGTACCG ACTGGTTATG TTGACACTGA AAATAACACA GAATTCACCA
5   GAGTTAATGC AAAACCTCCA CCAGGTGACC AGTTTAAACA TCTT,
SEQ ID 14: TCAGCCCCAG ATGGTACTTC TATTACCTAG GAACTGGCCC
AGAAGCTTCA CTT,
SEQ ID 23: TGCTCCAAGT GCCTCTGCAT TCTTTGGAAT GTCACGCATT
GGCATGGAAG TCACACCTT, or
10   SEQ ID 31: TGCCTATATG GAAGAGCCCT AATGTGTAAA ATTAATTTTA
GTAGTGCTAT CCCCATGTGA TTTTAATAGC TT,
or the complementary sequence thereof,
        a second oligonucleotide being 10-50 nucleotides in length
and comprising at least a fragment of 10 nucleotides of:
15   SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA
CCCGCGAAGA AGCTATTCGT CACGTTCGTG CGTGGATTGG CTTTGATGT,
SEQ ID 17: AGGTTTACCC AATAATACTG CGTCTTGGTT CACAGCTCTC
ACTCAGCATG GCAAGGAGGA ACTTAGATTC CCTCGAGGCC AGGGCGTTCC
AATCAACACC AATAGTGGTC CAGATGACCA AAT,
20   SEQ ID 26: CCAAACTGTC ACTAAGAAAT CTGCTGCTGA GGCATCTAAA
AAGCCTCGCC AAAAACGTAC TGCCACAAAA CAGTACAACG TCACTCAAGC
ATTTGGGAGA CGTGGTCCAG AACAAACCCA AGGAAATT, or
SEQ ID 34: TACGATACAT AGTCTACTCT TGTGCAGAAT GAATTCTCGT
AACTAAACAG CACAAGTAGG TTTAGTTAAC TTTAATCTCA CATAGCAATC
25   TTTAATCAAT GT,
or the complementary sequence thereof.
        More specifically, the pair of oligonucleotides, according
to the above definition, consists essentially of:
        a first oligonucleotide comprising, at least a fragment of
30   10 nucleotides, of a sequence selected from the group
consisting of:
SEQ ID 3: TCCACCAGGT GACCAGTTTA AACATCTT,
SEQ ID 4: TAGTAGCTGT ACCGACTGGT TATGTT,
SEQ ID 5: TACCTCTCCA GCTAGGATTT TCT,
35   SEQ ID 15: TCAGCCCCAG ATGGTACTTC T,

SEQ ID 16: TAGGAACTGG CCCAGAAGCT TCACTT,

SEQ ID 24: TGCTCCAAGT GCCTCTGCAT TCTT,

SEQ ID 25: TTGGCATGGA AGTCACACCT T,

5 SEQ ID 32: TGCCTATATG GAAGAGCCC,

SEQ ID 33: TCCCCATGTG ATTTTAATAG CTT,

or the complementary sequence thereof, and

a second oligonucleotide comprising, at least a fragment

of 10 nucleotides, of a sequence selected from the group

10 consisting of:

SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,

SED ID 7: GAAGCTATTC GTCACGTTCG,

SEQ ID 8: TGCGTGGATT GGCTTTGATG T,

SEQ ID 18: AGGTTTACCC AATAATACTG CGT,

15 SEQ ID 19: AGATTCCCTC GAGGCCAGGG CGT,

SEQ ID 20: ATAGTGGTCC AGATGACCAA AT,

SEQ ID 27: CCAAACTGTC ACTAAGAAAT CTGCT,

SED ID 28: CTCAAGCATT TGGGAGACGT GGT,

SEQ ID 29: CAGAACAAAC CCAAGGAAAT T,

20 SEQ ID 35: TACGATACAT AGTCTACTCT TGT,

SED ID 36: TAACTAAACA GCACAAGTAG GT,

SEQ ID 37: TAGCAATCTT TAATCAATGT,

or the complementary sequence thereof.

Another aspect of the invention is a pair of

25 oligonucleotides, for use as a set in the amplification of a

target sequence located within the replicase gene of the genome

of SARS Coronavirus, said pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length

and comprising at least a fragment of 10 nucleotides of: SEQ ID

30 1:TACCTCTCCA GCTAGGATTT TCTACAGGTG TTAACTTAGT AGCTGTACCG

ACTGGTTATG TTGACACTGA AAATAACACA GAATTCACCA GAGTTAATGC

AAAACCTCCA CCAGGTGACC AGTTTAAACA TCTT, or the complementary

sequence thereof, and

a second oligonucleotide being 10-50 nucleotides in length

35 and comprising at least a fragment of 10 nucleotides of:

SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA
CCCGCGAAGA AGCTATTCGT CACGTTCGTG CGTGGATTGG CTTTGATGT, or the
complementary sequence thereof.

5   More specifically, the pair of oligonucleotides, according
to the above definition, consists essentially of:
       a first oligonucleotide comprising at least a fragment of
10 nucleotides of a sequence selected from the group consisting
of:

10   SEQ ID 3:TCCACCAGGT GACCAGTTTA AACATCTT,
     SEQ ID 4:TAGTAGCTGT ACCGACTGGT TATGTT,
     SEQ ID 5:TACCTCTCCA GCTAGGATTT TCT,
     or the complementary sequence thereof, and
       a second oligonucleotide comprising at least a fragment of
15   10 nucleotides of a sequence selected from the group consisting
     of:
     SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,
     SED ID 7: GAAGCTATTC GTCACGTTCG,
     SEQ ID 8: TGCGTGGATT GGCTTTGATG T,
20   or the complementary sequence thereof.
       Another aspect of the invention is a pair of
oligonucleotides, for use as a set in the amplification of a
target sequence located within the gene encoding the
Nucleocapsid protein of the genome of SARS Coronavirus, said
25   pair consisting of:
       a first oligonucleotide being 10-50 nucleotides in length
and comprising at least a fragment of 10 nucleotides of:
SEQ ID 14: TCAGCCCCAG ATGGTACTTC TATTACCTAG GAACTGGCCC
AGAAGCTTCA CTT, or the complementary sequence thereof, or
30   a first oligonucleotide being 10-50 nucleotides in length
and comprising at least a fragment of 10 nucleotides of: SEQ ID
23: TGCTCCAAGT GCCTCTGCAT TCTTTGGAAT GTCACGCATT GGCATGGAAG
TCACACCTT, or the complementary sequence thereof, and
       a second oligonucleotide being 10-50 nucleotides in length
35   and comprising at least a fragment of 10 nucleotides of:

SEQ ID 17: AGGTTTACCC AATAATACTG CGTCTTGGTT CACAGCTCTC
ACTCAGCATG GCAAGGAGGA ACTTAGATTC CCTCGAGGCC AGGGCGTTCC
AATCAACACC AATAGTGGTC CAGATGACCA AAT, or the complementary

5   sequence thereof or,

     a second oligonucleotide being 10-50 nucleotides in length
and comprising at least a fragment of 10 nucleotides of:
SEQ ID 26: CCAAACTGTC ACTAAGAAAT CTGCTGCTGA GGCATCTAAA
AAGCCTCGCC AAAAACGTAC TGCCACAAAA CAGTACAACG TCACTCAAGC

10   ATTTGGGAGA CGTGGTCCAG AACAAACCCA AGGAAATT, or the complementary
sequence thereof.

     More specifically, the pair of oligonucleotides, according
to the above definition, consists essentially of:

     a first oligonucleotide comprising at least a fragment of

15   10 nucleotides of a sequence selected from the group consisting
of:

SEQ ID 15: TCAGCCCCAG ATGGTACTTC T,

SEQ ID 16: TAGGAACTGG CCCAGAAGCT TCACTT,

SEQ ID 24: TGCTCCAA GTGCCTCTGC ATTCTT,

20   SEQ ID 25: TTGGCATGGA AGTCACACCT T,

or the complementary sequence thereof, and

     a second oligonucleotide comprising at least a fragment of
10 nucleotides of a sequence selected from the group consisting
of:

25   SEQ ID 18: AGGTTTACCC AATAATACTG CGT,

SED ID 19: AGATTCCCTC GAGGCCAGGG CGT,

SEQ ID 20: ATAGTGGTCC AGATGACCAA AT,

SEQ ID 27: CCAAACTGTC ACTAAGAAAT CTGCT,

SED ID 28: CTCAAGCATT TGGGAGACGT GGT,

30   SEQ ID 29: CAGAACAAAC CCAAGGAAAT T,

or the complementary sequence thereof.

     Another aspect of the invention is a pair of
oligonucleotides, for use as a set in the amplification of a
target sequence located within the 3'-Non Coding Region (3'-

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NCR) of the genome of SARS Coronavirus, said pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 31: TGCCTATATG GAAGAGCCCT AATGTGTAAA ATTAATTTTA GTAGTGCTAT CCCCATGTGA TTTTAATAGC TT, or the complementary sequence thereof, and

a second oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID 34: TACGATACAT AGTCTACTCT TGTGCAGAAT GAATTCTCGT AACTAAACAG CACAAGTAGG TTTAGTTAAC TTTAATCTCA CATAGCAATC TTTAATCAAT GT, or the complementary sequence thereof.

More specifically, the pair of oligonucleotides, according to the above definition, consists essentially of:

a first oligonucleotide comprising at least a fragment of 10 nucleotides of a sequence selected from the group consisting of:

SEQ ID 32: TGCCTATATG GAAGAGCCC,

SEQ ID 33: TCCCCATGTG ATTTTAATAG CTT,

or the complementary sequence thereof, and

a second oligonucleotide comprising at least a fragment of 10 nucleotides of a sequence selected from the group consisting of:

SEQ ID 35: TACGATACAT AGTCTACTCT TGT,

SED ID 36: TAACTAAACA GCACAAGTAG GT,

SEQ ID 37: TAGCAATCTT TAATCAATGT,

or the complementary sequence thereof.

The oligonucleotide pair, according to any of the above definitions, may be constituted by a first oligonucleotide provided with a promoter sequence recognized by a DNA dependent RNA polymerase.

For instance, the sequences SEQ ID 9-11 and SEQ ID 39-44 actually comprise the complementary and reverse sequence as reflected by SEQ ID 3-5 and SEQ ID 15, 16, 24, 25, 32 and 33

respectively. In SEQ ID 9-11 and SEQ ID 39-44, the
complementary and reverse sequences of SEQ ID 3-5 and SEQ ID
15, 16, 24, 25, 32 and 33 are operably linked to a promoter
sequence (the T7 promoter sequence). This makes the sequences
5   especially suitable for use as downstream primer in a
transcription based amplification technique such as NASBA.

One of the oligonucleotides may serve as an "upstream
oligonucleotide", i.e., an upstream-primer, while the second
10   oligonucleotide serves as a "downstream oligonucleotide", i.e.
downstream primer, in the amplification reaction. The location
on the SARS-genome (or the sequence complementary thereto) to
which both oligonucleotides comprised in such a pair according
to the invention can anneal, will together define the sequence
15   of the nucleic acid that is amplified. The amplified sequence
is located between the "primer-binding sites" within the SARS
genome. It has been found that, by using a pair of
oligonucleotides according to the invention in an amplification
reaction, accurate and reliable amplification of nucleic acid
20   of SARS can be achieved.

A preferred pair of oligonucleotides according to the
invention in the SARS-CoV REP consist of a first primer
comprising the sequence of SEQ ID NO 4 or 10 and a second
primer with the sequence of SEQ ID NO 7, or of a second set
25   composed by a first primer comprising the sequence of SEQ ID NO
10 and by a second primer with the sequence of SEQ ID NO 8, or
of a third set composed by a first primer comprising the
sequence of SEQ ID NO 9 and by a second primer with the
sequence of SEQ ID NO 8. For use in a transcription based
30   amplification method, especially the NASBA amplification, the
oligonucleotide with SEQ ID NO 10 is preferred, in combination
with an oligonucleotide with the sequence of SEQ ID NO 7.

A most preferred pair of oligonucleotides according to the
invention in the SARS-CoV N:

35

in a first part of the nucleic acid sequence, hereafter called region 1, consists of a first primer comprising the sequence of SEQ ID NO 15 and a second primer with the sequence of SEQ ID NO 19, and

in a second part of the nucleic acid sequence, hereafter called region 2, consists of either

a first primer comprising the sequence of SEQ ID NO 41 and a second primer with the sequence of SEQ ID NO 28, or

a first primer comprising the sequence of SEQ ID NO 42 and a second primer with the sequence of SEQ ID NO 28, or

a first primer comprising the sequence of SEQ ID NO 42 and a second primer with the sequence of SEQ ID NO 29.

A most preferred pair of oligonucleotides according to the invention in the SARS-CoV 3'-NCR consist of a first set composed by a first primer comprising the sequence of SEQ ID NO 32 and by a second primer with the sequence of SEQ ID NO 35 and a second set composed by a first primer comprising the sequence of SEQ ID NO 33 and by a second primer with the sequence of SEQ ID NO 36.

Part of the oligonucleotides according to the invention are particularly suitable for use as a probe in the detection of nucleic acid amplified with a pair of oligonucleotides according to the invention. When used as a probe, said oligonucleotides may be provided with a detectable label.

Oligonucleotides according to the invention that are especially suitable as a probe consist of sequences of 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of:

SEQ ID 12: GTTCGTGCGT GGATTGGCTT TGATGTAGAG GGCTGTCATG CAACTAGAGA TGCTGT,

SEQ ID 21: GGCTACTACC GAAGAGCTAC CCGACGAGTT CGTGGTGGTG

ACGGCAAAAT GAAAGAGCTC AGCCCCAGAT GGTACTTCTA TTACCTAGGA

ACTGGCCCAG AAGCTTCACT TCCCTACGGC GCTAACAAAG AAGGCATCGT

5   ATGGGTTGCA ACTGAGGGAG CCTTGAATAC ACCCAAAGAC CACATTGGCA

CCCGCAATCC TAATAACAAT GCTGCCACCG TGCTACAACT TCCTCAAGGA

ACAACATTGC CAAAAGGCTT CTACGCAGAG GGAAGCAGAG GCGGCAGTCA

AGCCTCTTCT CGCTCCTCAT CACGTAGTCG CGGTAATTCA AGAAATTCAA

CTCCTGGCAG CAGTAGGGGA AATTCTCCTG CTCGAATGGC TAGCGGAGGT

10 GGTGAAACTG CCCTCGCGCT ATTGCTGCTA GACAGATTGA ACCAGCTTGA

GAGCAAAGTT TCTGGTAAAG GCCAACAACA ACAAGGCCAA ACTGTCACTA

AGAAATCTGC TGCTGAGGCA TCTAAAAAGC CTCGCCAAAA ACGTACTGCC

ACAAAACAGT ACAACGTCAC TCAAGCATTT GGGAGACGTG GTCCAGAACA

AACCCAAGGA AATTTCGGGG ACCAAGACCT AATCAGACAA,

15 SEQ ID 38: GCCACCACAT TTTCATCGAG GC,

or the complementary sequence thereof, provided with a

detectable label.

    A molecular beacon, preferably consisting of, constitutes

the probe:

20 SEQ ID 13: 5'-[6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-

[DabSyl]-3',

SEQ ID 45: 5'-[6-FAM]-cgcgatGTTCGTGCGTGGATTGGCTTatcgcg-

[DabCyl]-3',

SEQ ID 22: 5'-[6-FAM]-ccatgggCTACTACCGAAGAGCTACCCGACGAcccatgg-

25 [DabSyl]-3',

SEQ ID 30: 5'-[6-FAM]-ccatggACCAAGACCTAATCAGACAAccatgg-

[DabSyl]-3',

SEQ ID 47: 5'-[6-FAM]-ccatgcGCCACCACATTTTCATCGAgcatgg-[DabSyl]-

3'.

30   Various labeling moieties are known in the art. Said

moiety may, for example, either be a radioactive compound, a

detectable enzyme (e.g. horse radish peroxidase (HRP)), a

hapten like biotin, or any other moiety capable of generating a

detectable signal such as a colorimetric, fluorescent,

35 chemiluminescent or electrochemiluminescent signal.

Hybrids between oligonucleotides according to the invention and (amplified) target nucleic acid may also be detected by other methods known to those skilled in the art.

5   Evidently methods for amplification of nucleic acid, like the ones that have been mentioned above, using the oligonucleotides according to the present invention are also part of the invention.

The sequence in capital letters refers to the probe itself
10   according to SEQ ID 14 for instance, i.e. where the hybridisation between said probe and a part of the amplified nucleic acid takes place. Downstream and upstream extensions, in small letters, are sequences called here 7-nucleotide-long arm sequences that are able to hybridise one to the other.
15   These arm sequences relate to linkers between the probe and on the one hand a fluorophore (6-FAM) and on the other hand quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DabCyl or DabSyl). An ideal fluorophore-quencher pair is completely unable to fluoresce when the two components are in close
20   proximity, but as soon as the loop of the molecular beacon is conformationally changed due to hybridization of the probe to the amplified nucleic acid, the distance between the fluorophore and the quencher is sufficiently enlarged to permit emission of fluorescence.
25   Other fluorescent reporters are possible like 5' fluorescein, 5' TET, 5' HEX, 5' FAM, 5' TAMRA, 5' ROX, 5' Texas Red, 5' Oregon Green, 5' Cy3 or 5' Cy5. In the same way other quenchers, such as 3' Black Hole Quencher-1 or 3' Black Hole Quencher-2, can be used.
30   In another aspect of the invention, an oligonucleotides' pair, according to the ones here above disclosed, is used in a nucleic acid amplification reaction or as a probe for the detection of SARS Coronavirus nucleic acid in a sample. Consequently it is possible to perform a method for the
35   detection of SARS nucleic acid in a sample wherein the sample

is subjected to a nucleic acid amplification reaction using a
pair of oligonucleotides above disclosed and suitable
amplification reagents and the presence of any amplified

5   nucleic acid is detected. This method, wherein reacting the
sample with an oligonucleotide under suitable hybridization
conditions and detecting the presence of the label in any
hybrids could carry out the detection of any amplified nucleic
acid, formed between the amplified sequence and the probe.

10   Preferably the amplification technique used is a transcription
based amplification technique, more preferably the NASBA, and
the first oligonucleotide is provided with a promoter sequence
recognized by a DNA dependent RNA polymerase.
        The present invention further provides test kits for the

15   amplification and detection of SARS nucleic acid. The use of
said test kits enables accurate and sensitive screening of
samples suspected of containing SARS derived nucleic acid. Such
test kits may contain a pair of oligonucleotides according to
the invention and optionally also an oligonucleotide according

20   to the invention that can be used as a probe for the detection
of the amplified material. Furthermore the test kit may contain
suitable amplification reagents. These reagents are for example
the suitable enzymes for carrying out the amplification
reaction. A kit, adapted for use with NASBA, for example may

25   contain suitable amounts of reverse transcriptase, RNase H and
T7 RNA polymerase. Said enzymes may be present in the kit in a
buffered solution but can likewise be provided as a lyophilized
composition, for example, a lyophilized spherical particle.
Such lyophilized particles have been disclosed in

30   PCT/EP95/01268. The kit may further be furnished with buffer
compositions, suitable for carrying out an amplification
reaction. Said buffers may be optimized for the particular
amplification technique for which the kit is intended as well
as for use with the particular oligonucleotides that are

35   provided with the kit. In transcription-based amplification

techniques, such as NASBA, said buffers might contain, for
example, DMSO, which enhances the amplification reaction (as is
disclosed in PCT/US90/04733).

5   Furthermore the kit may be provided with an internal
control as a check on the amplification procedure and to
prevent the occurrence of false negative test results due to
failures in the amplification procedure. The use of internal
controls in transcription based amplification techniques is
10   described in PCT/EP93/02248. An optimal control sequence is
selected in such a way that it will not compete with the target
nucleic acid in the amplification reaction. Kits may also
contain reagents for the isolation of nucleic acid from
biological specimens prior to amplification. A suitable method
15   for the isolation of nucleic acid is disclosed in EP-A-
0.389.063.

Virus propagation and isolation from in-vitro cultures:
         A variety of clinical specimens (blood, serum, material
20   from oropharyngeal swabs or washings, material from
nasopharyngeal swabs, and tissues of major organs collected at
autopsy) were inoculated onto a number of continuous cell
lines, including Vero E6, NCIH292, MDCK, LLC-MK2 and B95-8
cells. Two cell lines, Vero E6 cells and NCI-H292 cells,
25   inoculated with oropharyngeal specimens from patients with SARS
initially showed cytopathic effect. A rhinovirus was isolated
from the inoculated NCI-H292 cells, but further study suggested
that this virus was not associated with patients with SARS.
Examination of cytopathic-effect-positive VeroE6 cells by thin-
30   section electron microscopy revealed characteristic coronavirus
particles within the cisternae of the rough endoplasmic
reticulum and in vesicles. Extracellular particles were found
in large clusters and adhering to the surface of the plasma
membrane. Vero E6 cells have now become the standard cell line
35   for the isolation and propagation of the SARS related human

Coronavirus (Marra *et al.* (1), Drosten *et al.* (10), Ksiazek *et al.* (13)).

Identification of specific nucleic acid fragments of the SARS
5   related human Coronavirus:
This identification has already been disclosed previously
in the disclosure according to a publication (Drosten *et al.*
(10)). A group at the National Microbiology Laboratory in
Canada also obtained a SARS genomic sequence (Genbank Accession
10   AY274119.3) from an isolate referred to as Tor2. The latter
sequence is essentially identical to the one that was released
independently by the CDC (GenBank accession number AY278741).
Additional bases in the Tor2 sequence that correspond to the 3′
(encoded) polyA tail were reported. Eight base differences
15   between the two sequences could represent sequencing errors,
PCR artefacts or mutable sites in the genome.(Marra *et al.*
(6)). The genome of SARS-Coronavirus is a 29,727-nucleotides
polyadenylated RNA, and 41% of the residues are G or C The
genomic organization is typical of coronaviruses, with the
20   characteristic gene order. The SARS-Coronavirus replicase gene,
which comprises approximately two-thirds of the genome, is
predicted to encode two polyproteins (encoded by ORF1a and
ORF1b) that undergo co-translational proteolytic processing.
There are four open reading frames (ORFs) downstream of
25   replicase that are predicted to encode the structural proteins
S, E, M, and N, which are common to all known coronaviruses.
The hemagglutinin-esterase (HE) gene, which is present between
ORF1b and S in group 2 and some group 3 coronaviruses, was not
found. (Rota *et al.* (7)). This is in agreement with the
30   Electron microscopic observations of virus particles lacking
the HE surface projections.
Phylogenetic analyses of the sequence of SARS-Coronavirus
indicate that SARS-Coronavirus is not closely related to any of
the previously characterized coronaviruses and forms a distinct
35   group within the genus *Coronavirus*. The SARS-Coronavirus is

approximately equidistant from all previously characterized coronaviruses, just as the existing groups are from one another. No evidence for recombination was detected when the predicted protein sequences were analyzed using SimPlot in comparison to sequences of known Coronaviruses.

The coronavirus subgenomic mRNAs are synthesized through a discontinuous transcription process, the mechanism of which has not been unequivocally established. The favoured model for production of subgenomic mRNAs of coronaviruses proposes that discontinuous transcription occurs during synthesis of the negative strand (Sawicki G. S. et al. (14)). The positions of the transcription regulating sequences (TRS) in the genome of SARS-Coronavirus predict that subgenomic mRNAs of 8.3, 4.5, 3.4, 2.5, 2.0, and 1.7 kb, not including the poly(A) tail, should be produced.

At least five subgenomic mRNAs were indeed detected by Northern hybridization of RNA from SARS-Coronavirus-infected cells, using a probe derived from the 3'-untranslated region. Full-length genomic RNA was not detected, likely because it is the least prevalent viral RNA in infected cells (Lai et al. (15)). The predicted 2.0 kb transcript was also not detected, suggesting that the consensus TRS at nucleotides 27,771–27,778 is not used or that it is a low-abundance mRNA (Rota et al. (2)).

The invention will further illustrated by the following non-limiting Examples, with reference to the accompanying Figures, in which:

Figures 1-4 show amplification curves of 4 different primer pairs ((Figure 1) SARS-CoV REP P1.1/P2.1, (Figure 2) SARS-CoV REP P1.1/P2.2, (Figure 3) SARS-CoV REP P1.2/P2.1 and (Figure 4) SARS-CoV REP P1.2/P2.2) tested on a dilution series of SARS Coronavirus in vitro REP RNA.

Figure 5 shows amplification curves of the selected primer pair (P1.1/P2.2), testing the analytical sensitivity of the SARS CoV NASBA.

Figure 6 shows the predicted secondary structures for SARS-CoV REP MB-1 and SARS-CoV REP MB-2.

Figures 7-11 show amplification curves of 5 other different primer pairs ((Figure 7) SARS-CoV REP P1.1/P2.3, (Figure 8) SARS-CoV REP P1.2/P2.3, (Figure 9) SARS-CoV REP P1.3/P2.1, (Figure 10) SARS-CoV REP P1.3/P2.2 and (Figure 11) SARS-CoV REP P1.3/P2.3) tested on a dilution series of SARS Coronavirus RNA.

Figure 12 show the predicted secondary structures for SARS-CoV N MB-1 and SARS-CoV N MB-2.

Figures 13-16 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 13) SARS-CoV N P1.1/P2.1; (Figure 14) SARS-CoV N P1.1/P2.2; (Figure 15) SARS-CoV N P1.2/P2.1; (Figure 16) SARS-CoV N P1.2/P2.2.

Figures 17-20 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV upon storage of the nucleic acid extracts prior to amplification. (Figure 17) SARS-CoV N P1.1/P2.1; (Figure 18) SARS-CoV N P1.1/P2.2; (Figure 19) SARS-CoV N P1.2/P2.1; (Figure 20) SARS-CoV N P1.2/P2.2.

Figures 21-24 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-2 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted

from a dilution series of cultured SARS-CoV. (Figure 21) SARS-CoV N P1.3/P2.3; (Figure 22) SARS-CoV N P1.3/P2.4; (Figure 23) SARS-CoV N P1.4/P2.3; (Figure 24) SARS-CoV N P1.4/P2.4.

5   Figures 25-28 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2. Four SARS-CoV N primer pairs (Table 6) were combined with molecular beacon SARS-CoV N MB-2 (Table 5) for the amplification and real-time detection of SARS-CoV RNA extracted
10   from a dilution series of cultured SARS-CoV upon storage of the nucleic acid extracts prior to amplification. (Figure 25) SARS-CoV N P1.3/P2.3; (Figure 26) SARS-CoV N P1.3/P2.4; (Figure 27) SARS-CoV N P1.4/P2.3; (Figure 28) SARS-CoV N P1.4/P2.4.

       Figures 29-34 show real-time amplification of SARS-CoV
15 viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Six SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 29) SARS-
20 CoV N P1.1/P2.1; (Figure 30) SARS-CoV N P1.1/P2.2; (Figure 31) SARS-CoV N P1.1/P2.5; (Figure 32) SARS-CoV N P1.2/P2.1; (Figure 33) SARS-CoV N P1.2/P2.2; (Figure 34) SARS-CoV N P1.2/P2.5.

       Figures 35-40 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2.
25 Six SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-2 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 35) SARS-CoV N P1.3/P2.3; (Figure 36) SARS-CoV N P1.3/P2.4; (Figure 37)
30 SARS-CoV N P1.3/P2.6; (Figure 38) SARS-CoV N P1.4/P2.3; (Figure 39) SARS-CoV N P1.4/P2.4; (Figure 40) SARS-CoV N P1.4/P2.6.

       Figure 41 show predicted secondary structures for the SARS-CoV 3'-NCR molecular beacon.

       Figure 42-47 show real-time amplification of SARS-CoV
35 viral RNA with SARS-CoV 3'-NCR primer/beacon mixtures. Six

SARS-CoV 3'-NCR primer pairs (Table 9) were combined with molecular beacon SARS-CoV 3'-NCR MB-1 (Table 10) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 42) SARS-CoV 3'-NCR P1.5/P2.5; (Figure 43) SARS-CoV 3'-NCR P1.5/P2.6; (Figure 44) SARS-CoV 3'-NCR P1.5/P2.7; (Figure 45) SARS-CoV 3'-NCR P1.6/P2.5; (Figure 46) SARS-CoV 3'-NCR P1.6/P2.6; (Figure 47) SARS-CoV 3'-NCR P1.6/P2.7.

Figures 48-51 show the analysis of SARS-CoV genomic RNA, strain Frankfurt, with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 48) SARS-CoV REP-1; (Figure 49) SARS-CoV REP-2; (Figure 50) SARS-CoV N-1; (Figure 51) SARS-CoV 3'-NCR.

Figures 52-55 show an analysis of SARS-CoV genomic RNA (Rotterdam preparation) with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 52) SARS-CoV REP-1; (Figure 53) SARS-CoV REP-2; (Figure 54) SARS-CoV N-1; (Figure 55) SARS-CoV 3'-NCR.

Figures 56-60 show an analysis of SARS-CoV genomic RNA (Rotterdam preparation) with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 56) SARS-CoV REP-1; (Figure 57) SARS-CoV REP-2; (Figure 58) SARS-CoV N-1; (Figure 59) SARS-CoV N-2; (Figure 60) SARS-CoV 3'-NCR.

EXAMPLES

First set of examples related to RNA issued from the viral
replicase gene:

Example 1: Materials and Methods:

1. SARS CoV *in vitro* RNA:

RNA encompassing 190 nucleotides of the SARS CoV replicase
gene was synthesized *in vitro* by transcription of a cloned
fragment of the viral gene, essentially as described in Drosten
et al. (10). The concentration of the *in vitro* generated RNA
was determined by OD (260 nm) measurement and appropriate
serial dilutions in water were stored at -70°C until further
use.

2. Oligonucleotides used for amplification and detection:

Primers and a molecular beacon detection probes (Tyagi, S.
and Kramer, F.R. (16)) were derived from the sequence of the
open reading frame (ORF) 1b of the SARS CoV replicase gene and
ordered as HPLC-purified oligonucleotides (Eurogentec,
Belgium). Sequence numbers and genome locations of the
oligonucleotides used in the amplification and of the molecular
beacon probe used for specific detection in real-time, are
shown in Table 1.

Table 1: Primers and molecular beacon probes for the amplification and detection in real-time of a region located in the replicase gene of SARS Coronavirus.

5

Primer/probe name	Sequence number	Primer/probe	Location
SARS-CoV REP P1.1	SEQ ID 10	Downstream primer 1.1	18319-18344
SARS-CoV REP P1.2	SEQ ID 11	Downstream primer 1.2	18283-18305
SARS-CoV REP P1.3	SEQ ID 9	Downstream primer 1.3	18389-18416
SARS-CoV REP P2.1	SEQ ID 6	Upstream primer 2.1	18153-18176
SARS-CoV REP P2.2	SEQ ID 7	Upstream primer 2.2	18201-18220
SARS-CoV REP P2.3	SEQ ID 8	Upstream primer 2.3	18221-18241
SARS-CoV REP MB-1	Label + SEQ ID 13 + quencher	Probe MB-1	18246-18271
SARS-CoV REP MB-2	Label + SEQ ID 45 + quencher	Probe MB-2	18216-18235

The two molecular beacons (SARS CoV MB-1 and MB-2) were designed for the detection of the amplicons that can be

10   generated with the SARS-CoV REP primers outlined in Table 1. Correct folding of the molecular beacons was checked using mfold (17) web server (http://www.bioinfo.rpi.edu/applications/mfold). Predicted secondary structures for SARS-CoV REP MB-1 and SARS-CoV REP MB-

15   2 are shown in Figure 6. Under NASBA reaction conditions, i.e.

41°C; 100 mM Na$^+$; 12 mM Mg^{2+}, both molecular beacons revealed the desired hairpin structure as the most favourable conformation. For SARS-CoV REP MB-1, additional hybrid

5 formation was predicted within the loop portion, resulting in two other possible conformations.

Molecular beacon syntheses were analysed for their purity by capillary electrophoresis (CE). Molecular beacons have a purity varying between 80,3% for SARS-CoV REP MB-2 and 88,7%

10 for SARS-CoV REP MB-1.

In figure 6, the arm sequences at the 5'-end and at the 3'-end are hybridized and here below depicted in small characters. Thus each probe is constituted as a molecular beacon, preferably consisting of:

15 SEQ ID 13: 5'-[6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-[DabSyl]-3', or

SEQ ID 45: 5'-[6-FAM]-cgcgatGTTCGTGCGTGGATTGGCTTatcgcg-[DabCyl]-3'.

Fluorophore 6-FAM is covalently linked to the 5'-end of

20 the molecular beacon; the quenching moiety DabSyl or DabCyl is covalently linked to the 3'-end of the molecular beacon. Coordinates for the location of the hybridising segments of the primers and the molecular beacon probe are derived from the complete genome sequence of SARS coronavirus TOR2 (EMBL

25 Accession number AY274119).

3. NASBA amplification with real-time detection:

RNA amplifications were performed using the NASBA amplification technology. To set up a NASBA amplification

30 reaction, NucliSens Basic Kit Amplification Reagents (bioMérieux bv, The Netherlands) were used. A premix was generated by reconstituting a Reagent Sphere in a mixture of 80 µl Reagent Sphere Diluent, 18 µl KCl stock solution and 12 µl NASBA Water. Subsequently, 10 µl of a mix containing two

35 primers for amplification and a molecular beacon probe for

real-time detection, were added, resulting in 2 x reaction buffer. Ten µl of this reaction buffer was added to 5 µl nucleic acid solution and incubated during 5 minutes at 65°C.

5 Then the reaction tubes were incubated at 41°C during 5 minutes. Meanwhile, an Enzyme Sphere was reconstituted in 55 µl Enzyme Diluent and 5 µl of the resulting enzyme mix was added into each reaction tube. After addition of the enzyme mix, tubes were mixed by gentle tapping, centrifuged briefly to

10 bring the contents to the bottom of the tubes and finally incubated at 41°C during 90 minutes in a NucliSens EasyQ Analyzer. Fluorescence in the individual reaction tubes was monitored over time and measured at regular intervals of 45 seconds.

15

Example 2: Selection of primer pair for the amplification of SARS CoV RNA:

To select a well performing primer pair for the

20 amplification of SARS CoV RNA using NASBA, three upstream primers, designated P2.1, P2.2 and P2.3, and three downstream primers , designated P1.1, P1.2 and P1.3, were derived from the sequence of the open reading frame (ORF) 1b of the SARS CoV replicase gene. Consequently, nine different primer pairs could

25 be formed, each consisting of a combination of one of the upstream primers with one of the downstream primers. With each of these primer pairs a standard dilution series of in vitro generated SARS CoV RNA was evaluated.

Primer pairs that can be formed with the SARS-CoV REP

30 primers (Table 1) are shown in Table 2 together with the size and genome location of the resulting amplicons. Results are shown in Figures 1-4 and Figures 7-11.

Table 2: SARS-CoV REP primer combinations:

Primer Combination	Amplicon Size	Genome Location
SARS-CoV REP P1.1/P2.1	192 nt	18,153-18,344
SARS-CoV REP P1.1/P2.2	144 nt	18,201-18,344
SARS-CoV REP P1.1/P2.3	124 nt	18,221-18,344
SARS-CoV REP P1.2/P2.1	153 nt	18,153-18,305
SARS-CoV REP P1.2/P2.2	105 nt	18,201-18,305
SARS-CoV REP P1.2/P2.3	85 nt	18,221-18,305
SARS-CoV REP P1.3/P2.1	264 nt	18,153-18,416
SARS-CoV REP P1.3/P2.2	216 nt	18,201-18,416
SARS-CoV REP P1.3/P2.3	196 nt	18,221-18,416

5

Figures 1-4 (for the first four pairs P1.1/P2.1, P1.1/P2.2, P1.2/P2.1 and P1.2/P2.2) show that all primer pairs revealed a positive result for the different amounts of in vitro RNA that were tested. However, analysis of the moment at which the different reactions start to reveal fluorescence signals that are well above the background signal showed that primer combination P2.2/P1.1 has strikingly lower Time-To-Positivity (TTP) values as the other primer combinations.

Therefore, oligonucleotide primers P1.1 and P2.2 were selected as the most ideal primer pair for the amplification of SARS CoV RNA.

5   In a next step, all nine SARS-CoV REP primer pairs (Table 2) were analysed. Since P1.3 was chosen outside the fragment covered by the *in vitro* RNA, these primer combinations were analysed on RNA extracted from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany). A nucleic

10   acid extract was prepared from 5 µl of this virus stock containing about 5000 genome equivalents (geq). From this nucleic acid extract a ten-fold dilution series in water was prepared containing nucleic acid levels as if extracted from 5000 geq (undiluted extract) down to 0.01 geq. Each of the nine

15   SARS-CoV REP primer combinations (see Table 2) was tested with this dilution series in real-time NASBA reactions using molecular beacon SARS-CoV REP MB-1 for detection. In addition to Figures 1-4, results are shown in Figure 7-11 for the other primer pairs (respectively for the following pairs P1.1/P2.3,

20   P1.2/P2.3, P1.3/P2.1, P1.3/P2.2 and P1.3/P2.3).

The four primer combinations previously tested on *in vitro* RNA, revealed a similar result with viral RNA extracted from the cultured SARS-CoV virus stock (Figures 1-4). Regarding sensitivity of the primer combinations, some minor differences

25   were observed, but whether these are significant, cannot be concluded on the basis of this limited evaluation. However, again primer pair P1.1/P2.2 showed faster kinetics and shorter, time-to-positivity values as the other three primer combinations.

30   For some of the new primer combinations containing either P1.3 or P2.3, sensitivity was comparable to the sensitivity of primer combination SARS-CoV REP P1.1/P2.2. In addition, primer combination SARS-CoV REP P1.1/P2.3 (Figure 7) showed kinetics and time-to-positivity values similar to those of primer

35   combination P1.1/P2.2. Strikingly, primer combination SARS-CoV

REP P1.2/P1.3 (Figure 8) revealed a much lower sensitivity as any of the other primer pairs. Probably, this is due to the small size of only 85 nts for the amplicons generated by this
5   primer pair (see Table 2). For NASBA amplification, an amplicon size below 100 nts is considered sub optimal. With 264 nts, primer pair SARS-CoV REP P1.3/P2.1 revealed the longest amplicons. This might explain the significantly lower kinetics, especially for the lower input levels, observed for this primer
10   combination (Figure 9).

From the nine possible primer combinations, more or less arbitrarily, primer combination SARS-CoV REP P1.1/P2.2 was selected for further evaluation and in combination with molecular beacon SARS-CoV REP MB-1 will be referred to as
15   primer/beacon mix SARS-CoV REP-1.

Another primer combination of interest in this region of the SARS-CoV genome, primer pair SARS-CoV REP P1.3/P2.3 in combination with molecular beacon SARS-CoV REP MB-1 was selected as SARS-CoV REP-2 for further studies.

20

Example 3: Analytical sensitivity of the selected primer pair:

To determine the analytical sensitivity of the selected primer pair (P1.1/P2.2) a dilution series of SARS CoV in vitro
25   RNA was tested with the selected primer pair. The RNA dilution series was made in NASBA water from a $1x10^5$ copies/μl RNA stock solution. Results are summarized in Figure 5 and show a very good analytical sensitivity with positive results down to 2.5 copies of in vitro RNA in de amplification, while the negative
30   control remained negative. In present evaluation, sensitivity of the selected primer pair (P1.3/P2.3) appeared somewhat less (one ten-fold dilution) as for the selected primer pair (P1.1/P2.2), but this is not considered to be a significant difference.

35

Example 4: SARS CoV REP-1 "ECL" version:

Based on the primers SARS-CoV P1.1.P2.2 primers and
molecular beacon MB-1, primers and a capture probe were
designed that could be used to perform real-time NASBA
amplification and subsequent end-point detection by
ElectroChemiLuminescence hereafter called ECL (18). Therefore,
the Basic Kit ECL-tail (Deiman, B. *et al.* (19)) was added to
the 5'-end of SARS-CoV REP P2.2 and the hybridizing fragment of
molecular beacon SARS-CoV REP MB-1 was synthesized as a
biotinylated oligonucleotide resulting in SARS-CoV REP 2.2
"ECL" and SARS-CoV REP Cap-1 (Capture probe), respectively.
Sequences, polarity, and genome locations of SARS-CoV REP-1
"ECL" are outlined in Table 3. For the P1 primer (SARS-CoV REP
P1.1) the T7 polymerase promoter sequence is depicted in small
characters. For the P2 primer (SARS-CoV REP P2.2 "ECL") the ECL
tail sequence added at the 5'-end of the primer is depicted in
small characters. The SARS-CoV Capture probe carries a biotin
moiety at its 5'-end. Coordinates for the location of the
hybridizing segments of the primers and the capture probe are
derived from the genome sequence of SARS-CoV strain TOR2
(EMBL/GenBank Accession Number AY274119).

Table 3: Primers and biotinylated capture probe for the
amplification and ECL-based end-point detection of a region
located in the replicase gene of SARS-CoV.

Description	Sequence (SEQ ID number)	Length	Genome location	Purity (CE)
SARS-CoV REP P.1.1	5'- aattctaatacgactcactatagggAACATAACCAG TCGGTACAGCTACTA-3' (SEQ ID 4)	51 nt	18,319– 18,344	74,1%
SARS-CoV REP P2.2 ECL	5'-gatgcaaggtcgcatatgagGAAGCTATTCGTCA CGTTCG-3' (SEQ ID 7)	40 nt	18,201– 18,220	88,6%
SARS-CoV REP Cap-1	5'-(Biotin)-GCTGTCATGCAACTAGAGATG CTGT-3' (SEQ ID 46)	21 nt	18,246– 18,271	ND

Primer/beacon mix "ECL" (containing primers SARS-CoV REP
P1.1 and SARS-CoV REP P2.2 "ECL" in combination with SARS-CoV
REP MB-1) was tested in real-time NASBA reactions using a
dilution series of *in vitro* RNA (18) as input material in the
reactions. For comparison, the same dilution series was
amplified with the standard SARS-CoV REP-1 primer/beacon mix.
Subsequently, ECL detection was performed on the SARS-CoV REP
P1.1 and SARS-CoV REP P2.2 in combination with SARS-CoV REP MB-
1 reactions, allowing direct comparison of ECL end-point
detection versus real-time molecular beacon detection on the
same amplification reactions. Results are summarized in Table
4, reflecting fluorescence signals for SARS-CoV REP-1 and both
fluorescence signals and ECL counts for SARS-CoV REP-1 "ECL".
The typical "all-or-nothing" results as obtained for SARS-CoV
REP-1 were also obtained for the ECL-version of this
primer/beacon mix and sensitivity of both primer/beacon
mixtures is identical. Comparison of real-time versus ECL
results for SARS-CoV REP-1 "ECL" revealed a similar result: ECL
counts were either very low or "overrange" (> 10^7 counts) and

dilutions found positive with real-time detection were positive with ECL detection as well.

5   Table 4: Comparison of SARS-CoV REP-1 primer/beacon mixtures and real-time versus ECL detection.

SARS CoV REP-1	
RNA input (cps per reaction)	Fluorescence signal
10	9,86
5	9,45
1	9,36
0,5	9,53
0,1	9,02
0,05	8,98
0,01	1,19
No Template	1,19

SARS CoV REP-1 "ECL"		
RNA input (cps per reaction)	Fluorescence signal	ECL signal (counts)
10	8,61	10.000.001
5	8,87	10.000.001
1	8,97	10.000.001
0,5	9,18	10.000.001
0,1	8,67	10.000.001
0,05	8,69	10.000.001
0,01	1,15	171
No Template	1,17	172

In vitro RNA was amplified with primer/beacon mixtures
SARS-CoV REP-1 and SARS-CoV REP-1 "ECL". For subsequent ECL
detection, SARS-CoV REP Cap-1.was used in combination with the
generic ECL probe of the NucliSens Basic Kit.

Second set of examples related to RNA issued from the viral
gene encoding the nucleocapsid protein:

Example 5: SARS-CoV Nucleocapsid primers design:

The SARS-CoV Nucleocapsid (N) protein is encoded by the
open reading frame spanning nucleotides 28,120-29,388 on the
viral genome. Primers were designed for two regions in the
nucleocapsid gene, the first region encompassing nucleotides

28,251-28,485 (region 1) and the second region spanning
nucleotides 28,851-29,101 (region 2). For each region,
initially two P1 primers (SARS-CoV N P1.1 and P1.2 for region
5 1; SARS-CoV N P1.3 and P1.4 for region 2) and two P2 primers
(SARS-CoV N P2.1 and P2.2 for region 1; SARS-CoV N P2.3 and
P2.4 for region 2) were designed. In each of the regions an
additional P2 primer was chosen that hybridized closer to the
sequence targeted by the molecular beacon for that region
10 (SARS-CoV N P2.5 for region 1; SARS-CoV N P2.6 for region 2).
Consequently, smaller amplicons could be generated which might
have a positive impact on the sensitivity of SARS-CoV N
subgenomic RNA detection. Sequences, polarity, and genome
locations of all SARS-CoV N primers are shown in Table 5.

15

Table 5: Primers for the amplification in real-time of two
regions located in the Nucleocapsid gene of SARS-CoV.

Region 1

20

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV N P1.1	5'- aattctaatacgactcactatagggAGAAGTACCA TCT GGGGCTGA-3' (T7 + SEQ ID 15)	46 nt	28,433- 28,453	85,5%
SARS-CoV N P1.2	5'- aattctaatacgactcactatagggAAGTGAAGCT TCT GGGCCAGTTCCTA-3' (T7 + SEQ ID 16)	51 nt	28,460- 28,485	85,1%
SARS-CoV N P2.1	5'-AGGTTTACCCAATAATACTGCGT-3' (SEQ ID 18)	23 nt	28,251- 28,273	90,2%
SARS-CoV N P2.2	5'-AGATTCCCTCGAGGCCAGGGCGT-3' (SEQ ID 19)	23 nt	28,315- 28,337	91,5%
SARS-CoV N P2.5	5'-ATAGTGGTCCAGATGACCAAAT-3' (SEQ ID 20)	22 nt	28,352- 28,373	90,0%

Region 2

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV N P1.3	5'- aattctaatacgactcactatagggAAGAATGCAGAG GCA CTTGGAGCA-3' (SEQ ID 41)	49 nt	29,043– 29,066	82,1%
SARS-CoV N P1.4	5'- aattctaatacgactcactatagggAAGGTGTGACTT CCATGC CAA-3' (SEQ ID 42)	46 nt	29,081– 29,101	81,8%
SARS-CoV N P2.3	5'-CCAAACTGTCACTAAGAAATCTGCT-3' (SEQ ID 27)	25 nt	28,851– 28,875	87,5%
SARS-CoV N P2.4	5'-CTCAAGCATTTGGGAGACGTGGT-3' (SEQ ID 28)	23 nt	28,934– 28,956	82,8%
SARS-CoV N P2.6	5'-CAGAACAAACCCAAGGAAATT-3' (SEQ ID 29)	21 nt	28,958– 28,978	89,8%

For the P1 primers (SARS-CoV N P1.1, P1.2, P1.3 and P1.4)
the T7 Polymerase promoter sequence is depicted in small
characters. Coordinates for the location of the primers are
derived from the complete genome sequence of SARS-CoV isolate
TOR2 (GenBank Accession number AY274119).
With two P1 primers and three P2 primers, six different SARS-
CoV N primer combinations can be composed for each of the two
regions (Table 6).

Table 6: SARS-CoV N primers combinations.

Primer Combination for region 1	Amplicon Size	Genome Location
SARS-CoV N P1.1/P2.1	203 nt	28,251-28,453
SARS-CoV N P1.1/P2.2	139 nt	28,315-28,453
SARS-CoV N P1.1/P2.5	102 nt	28,352-28,453
SARS-CoV N P1.2/P2.1	235 nt	28,251-28,485
SARS-CoV N P1.2/P2.2	171 nt	28,315-28,485
SARS-CoV N P1.2/P2.5	134 nt	28,352-28,485

Primer Combination for region 2	Amplicon Size	Genome Location
SARS-CoV N P1.3/P2.3	216 nt	28,851-29,066
SARS-CoV N P1.3/P2.4	133 nt	28,934-29,066
SARS-CoV N P1.3/P2.6	109 nt	28,958-29,066
SARS-CoV N P1.4/P2.3	251 nt	28,851-29,101
SARS-CoV N P1.4/P2.4	168 nt	28,934-29,101
SARS-CoV N P1.4/P2.6	144 nt	28,958-29,101

Example 6: SARS-CoV Nucleocapsid molecular beacons design:

For the detection of the amplicons that can be generated

5 with the different SARS-CoV N primers outlined in Table 5, a
molecular beacon was designed for each of the two selected
target regions in the N gene. The molecular beacons were
designated SARS-CoV N MB-1 for the upstream (region 1) region
and SARS-CoV N MB-2 for the downstream region (region 2).

10 Correct folding of the molecular beacons was checked using
mfold (17). Predicted secondary structures for SARS-CoV N MB-1
and SARS-CoV N MB-2 are shown in Figure 12. The mfold web
server ((http://www.bioinfo.rpi.edu/applications/mfold/) was
used to predict the most preferred secondary structures for

15 molecular beacons SARS-CoV N MB-1 and SARS-CoV N MB-2. Under
NASBA reaction conditions [41°C; 100 mM Na$^+$; 12 mM Mg^{2+}], both
molecular beacons revealed the desired hairpin structure.
Molecular beacon syntheses were analysed for their purity by
capillary electrophoresis (CE). Molecular beacon features are

20 summarized in Table 7.

Table 7: Molecular beacon probes for the detection in real-time
of two regions located in the Nucleocapsid gene of SARS-CoV.

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV N MB-1	5'-(6-FAM)- ccatgggCTACTACCGAAGAGCTACCCG ACGAcccatgg-(DabSyl)-3' (label + SEQ ID22 + quencher)	39 nt	28,377- 28,401	88,2%
SARS-CoV N MB-2	5'-(6-FAM)- ccatggACCAAGACCTAATCAGACAAccatgg- (DabSyl)-3' (label + SEQ ID 30 + quencher)	32 nt	28,985- 29,004	75,5%

25

For the molecular beacons (SARS-CoV N MB-1 and MB-2) the arm sequences at the 5'-end and at the 3'-end are depicted in small characters. Fluorophore 6-FAM is covalently linked to the 5'-end of the molecular beacons; the quenching moiety DabSyl is covalently linked to the 3'-end of the molecular beacons. Coordinates for the location of the molecular beacon probes are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).

Example 7: Selection of primer pair/beacon combination for the amplification and detection of SARS CoV N RNA:

For each of the regions chosen for amplification in the SARS-CoV Nucleocapsid gene sequence, six different primer combinations can be composed with the primers shown in Table 5. In Table 6, all combinations are summarized, together with the size of the corresponding RNA amplicons.

Initially, four SARS-CoV N primer pairs for region 1 that could be formed with P1.1, P1.2, P2.1, and P2.2 (Table 6) were tested in real-time NASBA reactions using molecular beacon SARS-CoV N MB-1 labelled with FAM (Table 7) for detection. Input RNA for these reactions was prepared from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany) by extracting nucleic acid from a ten-fold dilution series containing 10,000 down to 0.1 geq of the virus. Results are shown in Figures 13-16. Combined with either of the P2 primers, SARS-CoV N P1.1 revealed a better performance as P1.2 (compare Figures 13 and 14 with Figures 15 and16), although not in terms of sensitivity but with respect to kinetics and time-to-positivity values for the different dilutions.

In a follow-up experiment, the different primer combinations were compared again. Similar nucleic acid extracts were used as input material although the isolates were not

prepared freshly as in the previous experiment (Figures 13-16), but had been stored at -70°C for a while. Results are summarized in Figures 17-20. For reasons not understood, poor
5 results were obtained for the nucleic acid extract originating from 100 geq. Nevertheless, relative comparison of the different primer combinations again identified P1.1 as the best performing P1 primer. Combination of this P1 primer with P2.2 appeared to be the most favourable primer pair because SARS-CoV
10 N P1.1/P2.2 was the combination capable of most efficiently detecting the nucleic acid extract resulting from 1 geq and to reveal a positive albeit low signal for the "100 geq" nucleic acid extract (Figure 18) that unexpectedly tested negative for the other primer combinations.

15   In a comparable way as for region 1, four primer combinations for region 2 that can be compiled from primers SARS-CoV N P1.3, P1.4, P2.3, and P2.4 (Table 6) were analysed, now using FAM-labelled molecular beacon SARS-CoV N MB-2 (Table 7) for real-time detection. Results for freshly prepared
20 nucleic acid extracts are shown in Figures 21-24. In contrast to the primer pairs for region 1 in which a P1 primer was the dominating factor, for region 2 a P2 primer, viz. SARS-CoV N P2.4, appeared to be the crucial primer. It revealed a much better performance as the other P2 primer (compare Figures 21
25 and 23, 22 and 24), while combination with either SARS-CoV N P1.3 or P1.4 did not make too much of a difference (see Figures 22 and 24). Although kinetics for the "10 geq" nucleic acid extract for SARS-CoV N P1.3/P2.4 were slower as for the SARS-CoV N P1.4/P2.4 primer combination, the low but evidently
30 positive signal for the next dilution, i.e. the "1 geq" nucleic acid extract, (Figure 22) pleads in favour of the former primer combination.

      Analysis of stored nucleic acid extracts containing genomic viral RNA with the region 2 primer combinations
35 confirmed the results as obtained for the fresh specimens. As

can be seen from Figures 25-28, again P2.4 appeared to be the key primer. Combination with either P1.3 or P1.4 revealed similar results although the nucleic acid extract resulting

5   from 1 geq was missed by the SARS-CoV N P1.3/P2.4 primer pair whereas the next dilution in line was well detected. With all four primer combinations, poor results were obtained for the "100 geq" nucleic acid extract, confirming the poor results for this isolate with the region 1 primer combinations (Figures 17-

10   20).

In conclusion, this first endeavour to come and get to a well performing primer pair for the detection of SARS-CoV subgenomic mRNA encoding the nucleocapsid protein revealed SARS-CoV N P1.1/P2.2 in region 1 and either SARS-CoV N

15   P1.3/P2.4 or SARS-CoV N P1.4/P2.4 in region 2 as the most likely candidates. Overall comparison of the three primer combinations identified SARS-CoV N P1.1/P2.2 (region 1) in combination with SARS-CoV N MB-1 as the most favourable primer pair, mainly based on the shape of the fluorescence curves and

20   the absolute fluorescence signals that could be obtained with this primer/beacon mixture. Therefore, this primer/beacon mixture was selected as SARS-CoV N-1 for further evaluation.

In an attempt to further improve the sensitivity of SARS-CoV N mRNA detection, an additional P2 primer was designed for

25   both regions, viz. SARS-CoV N P2.5 for region 1 and SARS-CoV N P2.6 for region 2 (Table 5). In either case, this new P2 primer was located closer to the P1 primer resulting in a smaller amplicon size as obtained for the initial primer combinations (Table 6).

30   The six SARS-CoV N primer pairs that could now be formed for each of the two regions (Table 6) were tested in real-time NASBA reactions using the FAM-labelled SARS-CoV N molecular beacon designed for each of the regions (Table 7) for detection. Input RNA for these reactions was prepared from a

35   cultured SARS-CoV virus stock (provided by Erasmus Medical

Center, Rotterdam, The Netherlands). Nucleic acid was extracted from a 100-fold dilution of the original virus stock. From the resulting nucleic acid extract, a ten-fold dilution series was

5   prepared containing nucleic acid as if extracted from 10^3-fold down to 10^9-fold dilutions of the original virus stock.

Results for the SARS-CoV N primer pairs in region 1 are shown in Figures 29-34. Remarkably, primer combinations containing the new SARS-CoV N P2.5 primer (Figures 31 and 34)

10   demonstrated a significantly lower sensitivity as the other primer pairs instead of an anticipated better performance. This was somewhat unexpected since this P2 primer revealed shorter amplicons (102 nts and 134 nts in combination with P1.1 or P1.2, respectively; see also Table 6) and, therefore, was

15   expected to result in more efficient amplification. Comparison of the remaining primer combinations confirmed the already observed superiority of SARS-CoV N P1.1 over SARS-CoV N P1.2. However, in contrast to earlier observations (Figures 13-20), now SARS-CoV N P2.1 appeared to be superior over SARS-CoV N

20   P2.2, which again would not be logical in terms of amplicon length (203 nts versus 139 nts for P2.1 and P2.2, respectively; see also Table 6). The overall conclusion of this comparison is twofold:

for each of the two P1 primers: the larger the amplicon size,

25   the better the performance of the underlying primer combination; and

P1.1 revealed a better performance as P1.2.

Results for region 2 including the additional P2.6 primer are shown in Figures 35-40. When combined with SARS-CoV N P1.3,

30   the new P2 primer did not reveal the anticipated higher sensitivity (Figure 37), but instead showed a somewhat poorer performance as the SARS-CoV N P1.3/P2.4 primer pair both in terms of sensitivity and in time-to-positivity values for the individual dilutions. However, in combination with the other P1

35   primer chosen in this region and resulting in primer pair SARS-

CoV N P1.4/P2.6, sensitivity indeed increased, reflected by the result obtained for the nucleic acid extract obtained for the 10^9 dilution of the virus stock that only tested positive with

5 this region 2 primer pair (Figure 40).

Next to SARS-CoV N P1.4/P2.6 and in line with previous observations (see Figures 21-28), also SARS-CoV N P1.3/P2.4 (Figure 36) and SARS-CoV N P1.4/P2.4 (Figure 39) again were identified as better performing primer combinations in this

10 region and again these primer combinations mutually were nearly indistinguishable.

Third set of examples related to RNA encompassing the 3'-non coding region (3'-NCR):

15

Example 8: SARS-CoV 3'-NCR primers design:

The subgenomic mRNA transcripts of SARS-CoV form a 3'-co-terminal nested set with the viral genome and, consequently,

20 have a common 3'-end sequence. Excluding the poly(A)-tail, this 3'-untranslated or non-coding region (3'-NCR) is 339 nucleotides in length and encompasses genomic positions 29,389-29,727.

Primers were designed for the amplification of fragments

25 of the 3'-NCR between genomic nucleotides 29,447-29,713. Sequences, polarity, and genomic locations of these SARS-CoV 3'-NCR primers are shown in Table 8.

Table 8: Primers for the amplification in real-time of a region located in the 3'-untranslated sequence of SARS-CoV.

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV 3'-NCR P1.5	5'-aattctaatacgactcactatagggGGGCTCTT CCATATA GGCA-3' (T7 + SEQ ID 32)	44 nt	29,642-29,660	83,3%
SARS-CoV 3'-NCR P1.6	5'-aattctaatacgactcactatagggAAGCTATT AAAATC ACATGGGGA-3' (T7 + SEQ ID 33)	48 nt	29,691-29,713	85,6%
SARS-CoV 3'-NCR P2.5	5'-TACGATACATAGTCTACTCTTGT-3' (SEQ ID 35)	23 nt	29,447-29,469	91,7%
SARS-CoV 3'-NCR P2.6	5'-TAACTAAACAGCACAAGTAGGT-3' (SEQ ID 36)	22 nt	29,486-29,507	86,5%
SARS-CoV 3'-NCR P2.7	5'-TAGCAATCTTTAATCAATGT-3' (SEQ ID 37)	20 nt	29,529-29,548	91,3%

5

For the P1 primers (SARS-CoV 3'-NCR P1.5 and P1.6) the T7 Polymerase promoter sequence is depicted in small characters. Coordinates for the location of the primers are derived from 10 the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).

Two P1 primers (SARS-CoV 3'-NCR P1.5 and P1.6) and three P2 primers (SARS-CoV 3'-NCR P2.5, P2.6, and P2.7) were derived from the viral genome sequence and enabled the composition of 15 six different primer pairs (Table 9).

Table 9: SARS-CoV 3'-NCR primer combinations.

Primer Combination	Amplicon Size	Genome Location
SARS-CoV 3'-NCR P1.5/P2.5	214 nt	29,447-29,660
SARS-CoV 3'-NCR P1.5/P2.6	175 nt	29,486-29,660
SARS-CoV 3'-NCR P1.5/P2.7	132 nt	29,529-29,660
SARS-CoV 3'-NCR P1.6/P2.5	267 nt	29,447-29,713
SARS-CoV 3'-NCR P1.6/P2.6	228 nt	29,486-29,713
SARS-CoV 3'-NCR P1.6/P2.7	185 nt	29,529-29,713

5

Primer pairs that can be formed with the SARS-CoV 3'-NCR primers (Table 8) are given together with the size and genome location of the resulting amplicons.

10   Example 9: SARS-CoV 3'-NCR molecular beacon design:

Molecular beacon SARS-CoV 3'-NCR MB-1 was designed as shown in Table 10 for the detection of amplicons that can be generated with the SARS-CoV 3'-NCR primers outlined in Table 8.

Table 10: Molecular beacon for the detection in real-time of amplicons generated from a region located in the 3'-untranslated sequence of SARS-CoV.

5

Description	Sequence	Length	Genome Location	Purity (CE)
SARS-CoV 3'-NCR MB-1	5'-(6-FAM)-ccatgcGCCACCACATTTTCATCGAG gcatgg-(DabSyl)-3' (label + SEQ ID 47 + quencher)	32 nt	29,574-29,595	84,6%

For the molecular beacon (SARS-CoV 3'-NCR MB-1) the arm sequences at the 5'-end and at the 3'-end are depicted in small characters. Fluorophore 6-FAM is covalently linked to the 5'-
10   end of the molecular beacon; the quenching moiety DabSyl is covalently linked to the 3'-end of the molecular beacon. Coordinates for the location of the molecular beacon probe are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).
15   Correct folding of the molecular beacon was checked using mfold (17). The secondary structure as predicted for SARS-CoV 3'-NCR MB-1 is shown in Figure 41. Under NASBA reaction conditions [41°C; 100 mM Na$^+$; 12 mM Mg^{2+}], the molecular beacon revealed the desired hairpin structure.
20   Synthesis of the molecular beacon was analysed by capillary electrophoresis (CE) and revealed a purity of about 84.6% (Table 10).

Example 10: Selection of primer pair/beacon combination for the
25   amplification and detection of SARS-CoV 3'-NCR:

Six different primer combinations could be formed with the SARS-CoV 3'-NCR primers shown in Table 8. Primer combinations are depicted in Table 9 together with the size of the resulting
30   RNA amplicons.

The different SARS-CoV 3'-NCR primer pairs were combined with the FAM-labelled SARS-CoV 3'-NCR molecular beacon (Table 10) and tested in real-time NASBA reactions. Input RNA for these reactions was prepared from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany). From a nucleic acid extract prepared from 1000 geq of the virus, a ten-fold dilution series in water was prepared. This dilution series contained nucleic acid levels as if extracted from 100 geq down to 0.01 geq.

Results for the six primer combinations are shown in Figures 42-47 and do not identify a preferred P1 or P2 primer but rather two unrelated primer combinations being SARS-CoV 3'-NCR P1.5/P2.5 and P1.6/P2.6 (Figures 42 and 46 respectively). For the other primer combinations, a lower sensitivity (SARS-CoV 3'-NCR P1.5/P2.6; Figure 43) or poorer kinetics, especially for the lower input levels (Figures 44, 45 and 47), were observed.

Showing "steeper" and faster (i.e. shorter TTP values) kinetics, primer pair SARS-CoV 3'-NCR P1.6/P2.6 was chosen for further evaluation and in combination with molecular beacon SARS-CoV 3'-NCR MB-1 will be referred to as primer/beacon mix SARS-CoV 3'-NCR.

Comparison of SARS-CoV primer/molecular beacon mixtures for different regions of the genome:

Upon analysis of several different primer combinations in three regions of the viral genome, viz. the replicase gene, the nucleocapsid gene, and the untranslated 'leader' sequence at the 3'-end of the genome, the best performing primer/beacon mixtures for each of the regions were selected for mutual comparison. In Table 11 these primer/beacon mixtures and their individual components are summarized.

Table 11: SARS-CoV primer/beacon mixtures targeting different regions on the SARS-CoV viral genome and their individual constituents.

5

Primer/beacon mix	P1 primer	P2 primer	Molecular beacon
SARS-CoV REP-1	SARS-CoV REP P1.1	SARS-CoV REP P2.2	SARS-CoV REP MB-1
SARS-CoV REP-2	SARS-CoV REP P1.3	SARS-CoV REP P2.3	SARS-CoV REP MB-1
SARS-CoV N-1	SARS-CoV N P1.1	SARS-CoV N P2.2	SARS-CoV N MB-1
SARS-CoV N-2	SARS-CoV N P1.4	SARS-CoV N P2.6	SARS-CoV N MB-2
SARS-CoV 3'-NCR	SARS-CoV 3'-NCR P1.6	SARS-CoV 3'-NCR P2.6	SARS-CoV 3'-NCR MB-1

Example 11: Cultured SARS-CoV virus strains – first sample:

A first comparison of the different primer/beacon mixtures

10   was made on viral genomic RNA extracted from a cultured SARS-CoV specimen that was kindly provided by the Robert Koch Institute in Berlin, Germany. This cultured SARS-CoV virus originated from the SARS case cluster from Frankfurt, Germany (10). Based on this virus stock with a known concentration of

15   virus particles (in genome equivalents (geq) per ml), a tenfold dilution series was prepared. RNA was extracted in duplicate from 2000 geq down to 0.2 geq of the virus using SAME extraction (see "Materials and Methods" section) and finally eluted in 50 µl elution buffer. Consequently, RNA resulting

20   from 200 geq down to 0.02 geq was amplified with five different primer/beacon mixtures that were selected for further evaluation (Table 11).

Typical analyses are shown in Figures 48-51 and overall results are summarized in Table 12.

Table 12: Analysis of SARS-CoV genomic RNA, strain Frankfurt,
5   with different primer/beacon mixtures (Results from two analyses were compiled).

RNA in ampl. a (geq)	SARS-CoV REP-1	SARS-CoV REP-2	SARS-CoV N-1	SARS-CoV 3'-NCR
200	2/2	2/2	2/2	2/2
20	2/2	2/2	2/2	2/2
2	2/2	2/2	2/2	1/2
0.2	2/2	2/2	2/2	2/2
0.1	1/2	0/2	2/2	0/2
0.02	0/2	0/2	0/2	0/2

a = assuming 100% recovery in nucleic acid extraction

10

SARS-CoV N-1 and SARS-CoV REP-1 and REP-2 primer/beacon mixtures revealed the highest sensitivity. SARS-CoV N-1 was the most sensitive combination although differences with the SARS-CoV REP mixtures were minimal. SARS-CoV 3'-NCR showed
15   intermediate sensitivity.

Example 12: Cultured SARS-CoV virus strains – second sample:

For a second comparison, cultured virus from a different
20   source was used. This virus stock originated from the Erasmus Medical Center in Rotterdam, The Netherlands. Concentration of virus particles in this virus preparation was unknown. Therefore, a limiting dilution series was prepared ranging from a 100-fold dilution of the original virus culture down to a
25   10^9-fold dilution. RNA was extracted from each dilution using SAME extraction (see "Materials and Methods" section) and

amplified with each of the selected primer/beacon mixtures (Table 11). Typical analyses are shown in Figures 52-55 and overall results of three dilution series are summarized in Table 13)

Table 13: Analysis of SARS-CoV genomic RNA, strain Rotterdam, with different primer/beacon mixtures (Results from three analyses were compiled).

Virus stock dilution Factor	SARS-CoV REP-1	SARS-CoV REP-2	SARS-CoV N-1	SARS-CoV 3'-NCR
10^2	3/3	3/3	3/3	3/3
10^3	3/3	3/3	3/3	3/3
10^4	3/3	3/3	3/3	3/3
10^5	3/3	3/3	3/3	3/3
10^6	3/3	3/3	3/3	3/3
10^7	2/3	3/3	3/3	3/3
10^8	1/3	1/3	2/3	1/3
10^9	1/3	0/3	0/3	0/3
Neg	0/3	0/3	0/3	0/3

In line with the results found for the virus from Frankfurt, again SARS-CoV N-1 and SARS-CoV REP primer/beacon mixtures turned out to be the most sensitive combinations. Looking at the lower virus concentrations (10^7-10^9 dilutions), SARS-CoV N-1 revealed the most consistent results. However, SARS-CoV REP-1 was the only primer/beacon mixture to detect the 10^9-fold dilution albeit in only one out of three tests.

Example 13: Cultured SARS-CoV virus strains – third sample:

For a third comparison, again cultured virus from the
Erasmus Medical Center in Rotterdam, The Netherlands, was used.
5 Concentration of virus particles in this virus preparation was
unknown. Therefore, a limiting dilution series was prepared
ranging from a 10^3-fold dilution of the original virus culture
down to a 10^{10}-fold dilution. RNA was extracted from each
dilution using SAME extraction (see "Materials and Methods"
10 section) and amplified with each of the selected primer/beacon
mixtures (Table 11). Analyses are shown in Figures 56-60.
In this comparison, SARS-CoV REP-1 (Figure 56), SARS CoV REP-2
(Figure 57) and SARS-CoV N-2 (Figure 59) appeared to be the
most sensitive primer/beacon mixtures. However, the differences
15 with SARS CoV N-1 (Figure 58) was only one 10-fold dilution.
SARS CoV 3'-NCR (Figure 60) again revealed the lowest
sensitivity as was also observed in the other comparisons that
were made on cultured viral materials.

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